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Citation for published version:

Minchin, JEN & Rawls, JF 2017, 'Elucidating the role of plexin D1 in body fat distribution and susceptibility to metabolic disease using a zebrafish model system', *Adipocyte*, pp. 1-7.
<https://doi.org/10.1080/21623945.2017.1356504>

Digital Object Identifier (DOI):

[10.1080/21623945.2017.1356504](https://doi.org/10.1080/21623945.2017.1356504)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Adipocyte

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Elucidating the role of Plexin D1 in body fat distribution and susceptibility to metabolic disease using a zebrafish model system

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Abstract

Non-communicable diseases (NCDs) such as cardiovascular disease, diabetes and cancer were responsible for 68% of all deaths worldwide in 2012. The regional distribution of lipid deposited within adipose tissue (AT) - so called body fat distribution (BFD) - is a strong risk factor for NCDs. BFD is highly heritable; however, the genetic basis of BFD is almost entirely unknown. Genome-wide association studies have identified several loci associated with BFD, including at Plexin D1 (*PLXND1*) - a gene known to modulate angiogenesis. We recently demonstrated that zebrafish homozygous for a null mutation in *plxnd1* had a reduced capacity to store lipid in visceral AT (VAT) leading to altered BFD. Moreover, we found that type V collagens were upregulated in *plxnd1* mutants, and mediated the inhibitory effect of Plxnd1 on VAT growth. These results strengthen evidence that Plxnd1 influences BFD in human populations, and validate zebrafish as a model to study BFD. However, many pertinent questions remain unanswered. Here we outline potential Plxnd1 mechanisms of action in AT, and describe the genetic architecture at human *PLXND1* that is associated with BFD and NCD susceptibility.

Commentary

Adipose tissues (ATs) regulate energy homeostasis by supplying and sequestering energy-dense lipid in response to fluctuations in energy status. As such, AT provides an organism with energetic stability [1]. Evolutionarily, the energy insurance provided by AT confers tremendous selective advantages for a population when confronted with diverse physiological burdens. However, in modern societies - when energy-dense food is readily available, food consumption is high and physical activity is low - excessive lipid deposition within AT can lead to AT dysfunction and systemic metabolic disturbance increasing risk for non-communicable diseases (NCDs) such as cardiovascular disease, diabetes and cancer. In 2012, NCDs accounted for 68% of all deaths worldwide [2, 3]. Of the 52.8 million deaths globally in 2010, ischemic heart disease and stroke collectively killed 12.9 million (24% of all deaths), 1.3 million deaths were caused by diabetes (2.5%) and 8 million died from cancer (15%). Therefore, understanding factors that influence or predict NCD risk is an important public health challenge.

38 ATs are highly heterogeneous and deposited in diverse regional locations throughout the
39 body. Regionally distinct ATs have unique molecular and metabolic attributes that influence
40 whole-animal physiology. Accumulation of AT in the upper body (an android BFD) is associated
41 with increased risk for NCDs [4]. Whereas accumulation of AT in the lower body, primarily the
42 legs and thighs (a gynoid BFD) protects from NCD risk [4]. Android BFD is characterized by
43 increases in visceral AT (VAT, AT within the abdominal cavity) and abdominal subcutaneous AT
44 (SAT, AT between skin and muscle), whilst gynoid BFD is characterized by increased gluteal
45 and femoral SAT [4]. Understanding factors that regulate the diverse patterns of BFD within
46 human populations is likely to provide important new therapeutic interventions for NCDs.

47 Heritability estimates from twin studies suggest that BFD is under extensive genetic
48 control [5, 6]. However, the genetic basis of BFD is essentially unknown. Recently, the Genetic
49 Investigation of ANthropometric Traits (GIANT) consortium performed large-scale meta-
50 analyses of genome-wide association studies (GWAS) to identify loci associated with waist-hip
51 ratio (WHR) – a surrogate measure of android and gynoid patterns of BFD [7, 8]. Intriguingly,
52 GWAS have found WHR-associated loci are independent from more generalized adiposity traits,
53 suggesting that a distinct genetic architecture underlies BFD [9]. GWAS provide an unbiased
54 and comprehensive assessment of genetic loci associated with WHR. However, functional
55 characterization of GWAS loci is essential to identify mechanisms influencing BFD and disease
56 susceptibility.

57 The rs10804591 single nucleotide polymorphism (SNP) identified in Shungin et al. (2015)
58 encodes a C → A base change, at 3q22.1 ~8kb upstream of the *PLXND1* transcriptional start
59 site (Fig. 1) [7]. The rs10804591 A allele (effect allele, EA) was associated with increased
60 WHRadjBMI (WHR adjusted for BMI) ($P = 2.31 \times 10^{-6}$), increased susceptibility to type 2 diabetes
61 ($P = 1.67 \times 10^{-3}$), increased fasting glucose ($P = 0.048$), increased fasting insulin ($P = 6.08 \times 10^{-3}$),
62 increased blood triglycerides ($P = 9.37 \times 10^{-4}$), decreased Adiponectin ($P = 7.81 \times 10^{-3}$),
63 increased risk for coronary artery disease ($P = 0.018$) and decreased height ($P = 2.53 \times 10^{-5}$).
64 Similar to many of the BFD-associated SNPs, rs10804591 demonstrated a high degree of sexual
65 dimorphism – often exerting a stronger effect in women (Fig. 2) [7]. Further, the effect in males
66 and females appeared different, with males also exhibiting reductions in both waist and hip
67 circumferences (Fig. 2) [7]. Gender differences in adiposity are well known, with females having
68 a higher body fat percentage, and greater gluteal-femoral AT relative to males [10]. BFD is
69 regulated by sex hormones, as evidenced by redistribution of AT towards an android distribution
70 following menopause [11, 12]. rs10804591 EA is common, present at a frequency of 28%, 65%,
71 28%, 78% within African, American, East Asian, and European populations respectively (1000G
72 Phase 1, a 51% frequency in all individuals). Intriguingly, rs10804591 is located within a
73 predicted promoter flank region 5' of *PLXND1* (asterisk in Fig. 1), suggesting that rs10804591
74 might regulate *PLXND1* expression. However, the mechanism of rs10804591 action is
75 completely unknown. Importantly, within individuals of European ancestry, rs10804591 is also
76 linked to 41 other common SNPs clustered 5' to *PLXND1* ($>0.7 R^2$ linkage disequilibrium) (Fig.
77 1), and many of these linked SNPs also reside in predicted regulatory regions (Fig. 1). Searching
78 the Genotype-Tissue Expression (GTEx) Project revealed that the majority of the 41 SNPs at
79 *PLXND1* were associated with *PLXND1* mRNA changes in whole blood (N = 338). No

associations were found with expression changes in VAT (N = 185); however, this is potentially due to lower sample size. The investigation of functional variants at *PLXND1* is likely to provide exciting new insights into the genetic underpinnings of BFD.

PLXND1 is a multipass transmembrane receptor for a variety of Semaphorin (SEMA) ligands, including SEMA3E [13, 14] and SEMA4A [15]. Binding of SEMA3E/4A to *PLXND1* suppresses angiogenesis - the process of new blood vessel formation from existing vessels [13-15], and mutation of *Plxnd1* in mouse and zebrafish causes hypervascularization of multiple tissues [16, 17]. Therefore, *PLXND1* is a potent anti-angiogenic molecule. The role of angiogenesis is of particular relevance to AT biology as angiogenesis is known to regulate lipid accumulation in AT [18-20], and stimulation of angiogenesis specifically in AT can normalize metabolic disturbances present in obesity [20, 21]. Furthermore, depot-specific angiogenesis has been linked to systemic insulin resistance – a precursor to diabetes [22], suggesting that depot-specific differences in angiogenesis may underlie regional AT expansion and NCD progression. Further, we found that *PLXND1* mRNA was positively associated with hypertrophic morphology in VAT, and was increased in obese type 2 diabetics relative to lean and healthy obese subjects [23].

Prior to analysis of human *PLXND1*, we turned to zebrafish as a tractable in vivo model system to functionally evaluate the role of *Plxnd1* on BFD. Zebrafish possess AT that is morphologically, molecularly, and functionally homologous to mammalian white AT [23-30]. Further the molecular mechanisms governing AT dynamics seems conserved from zebrafish to mammals, as suggested by modulators of nuclear receptors exerting similar effects [31]. Fluorescent lipophilic dyes such as Nile Red and BODIPY can be utilized to visualize and quantify regional AT in live zebrafish (Fig. 3). Analysis of zebrafish homozygous for the functionally null *plxnd1* allele, *fov01b*, revealed an altered BFD, characterized by reduced VAT [23] (both pancreatic and abdominal VAT deposits) [30]. On closer inspection we found *plxnd1* mutant VAT was in a hyperplastic and hyperproliferative state, with an induction of type V collagens in vascular endothelial cells and altered extracellular matrix (ECM) composition [23]. Maintenance of the hyperplastic/hyperproliferative state was dependent on collagen type V alpha 1 (*col5a1*) and conferred resistance to VAT expansion coupled with improved glucose tolerance after exposure to a high-fat diet [23]. These data suggest that the ECM microenvironment can determine the proliferative capacity and growth of VAT, and that vascular endothelial cell-derived *Plxnd1* modulates the VAT ECM microenvironment in part through *Col5a1* (Fig. 4A). Regarding this mechanism, here we discuss a potential Integrin-mediated pathway by which *Plxnd1* may regulate ECM composition.

Integrins are heterodimeric collagen receptors that mediate cross-talk between the cell cytoplasm and extracellular ECM [32]. The Integrin family of genes is comprised of 18 distinct α subunits and 8 β subunits, which can dimerize to produce 24 heterodimeric combinations (the Integrin code). Integrin expression can be regulated transcriptionally [33-37], post-transcriptionally by miRNAs [38], and also at the post-translational level. For example, Integrins can form an inactive ‘closed confirmation’ with low affinity for extracellular ligands, or an active ‘open confirmation’ with high affinity for ligands. Regulation of these states has been well studied,

121 with multiple regulators identified (e.g., SHARPIN and SHANK) [39, 40]. Although how this form
122 of Integrin regulation impacts adipose tissue is currently unknown. Intriguingly, the metabolic
123 sensor, AMP-activated protein kinase (AMPK), was recently also identified as a regulator of β 1
124 Integrin activity [41]. However, a role for AMPK in regulating adipose ECM and growth is also
125 unknown. Distinct Integrin heterodimers possess different ECM-binding potentials [42], and
126 regulate ECM abundance and composition by modulating collagen synthesis and turnover [43,
127 44]. Therefore, we hypothesize that PlxnD1 modulates the collagen composition of VAT by
128 regulating the Integrin code displayed on the vascular endothelial cell-surface. It is known that
129 Integrin expression changes during adipocyte differentiation [45], and that overexpression of
130 Integrin α 5 in preadipocytes leads to enhanced proliferation and attenuated differentiation [45].
131 GTPases hydrolyze guanosine triphosphate (GTP), and the GTPase, Rac, is normally
132 downregulated during adipocyte differentiation [45]. Overexpression of Integrin α 5 increases
133 Rac activity, suggesting that GTPase levels are critical for preadipocyte proliferation and
134 differentiation [45, 46]. In support, Focal Adhesion Kinase (FAK) plays a central role in Integrin
135 signaling and is essential for adipose expansion [47, 48]. GTPases control many cell functions,
136 including deposition and maintenance of Integrins on the vascular endothelial cell surface [49-
137 53]. Plexin receptors are well known to regulate GTPase activity via their intracellular GTPase
138 activating-protein (GAP) domain [54], and recent studies demonstrated that binding of SEMA3E
139 to PLXND1 in Human Umbilical Vein Endothelial Cells (HUVECs), inactivated the GTPase
140 activity of R-Ras [14]. Work from the same lab further found that PLXND1 stimulated ARF6
141 GTPase activity by local production of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) by type I
142 phosphatidylinositol-4-phosphate-5-kinase (PIP5K) β [55]. Both of these pathways acted to
143 modulate Integrin presentation on the HUVEC surface [56]. The role of Integrins has not been
144 fully elucidated in AT [45, 57]. Further, the role of endothelial cell-localized Integrins on AT
145 formation and growth appears essentially unstudied. However, based on the established
146 mechanisms described above, we speculate that Integrin composition on the surface of VAT
147 endothelial cells may play an important role in PlxnD1-mediated regulation of BFD.

148 To test this hypothesis it will be necessary to manipulate the Integrin code on vascular
149 endothelial cells and assess effects on ECM and VAT growth. Such experiments may be
150 conducted by using the *Tie2-Cre^{Tg}* (*Tek-Cre*) transgenic mouse line [58] to produce vascular
151 endothelial cell-specific Integrin knockouts. Similar experiments have been performed previously
152 to assess an endothelial cell-specific role for β 1 Integrins [59-62]. As we hypothesize that β 1
153 Integrins mediate crosstalk between VAT endothelial cells and the ECM to regulate VAT growth
154 [55], it will be essential to temporally control Cre-mediated recombination due to embryonic
155 defects in β 1 Integrin knockout mice by using inducible Cre lines [59]. Although the conditional
156 knockout strategy described above allows the ablation of Integrins to be restricted to endothelial
157 cells, and further controlled by using inducible Cre lines, it would also be desirable to restrict
158 Integrin ablation to endothelial cells specifically within VAT. However, to our knowledge no such
159 transgenic line currently exists that expresses solely in VAT endothelial cells. Therefore, such
160 an experimental strategy will induce Integrin knockout in endothelial cells across the body,
161 potentially leading to secondary effects on VAT growth. Molecular profiles of tissue-specific
162 endothelial cells has been performed for a variety of tissue types [63], therefore a similar strategy
163 in adipose tissues may yield adipose-specific endothelial cell profiles that may be utilized for

transgenic strategies. However, to circumvent secondary effects, endothelial cell and adipocyte co-cultures may also need to be performed [64, 65].

Acknowledgements

This work was supported by American Heart Association Postdoctoral Fellowships (11POST7360004 and 13POST16930097), a University of Edinburgh and British Heart Foundation Fellowship, and a Diabetes UK grant (16/0005494) to JENM; and grants from NIH (R56-DK091356, R21-ES023369, R01-DK093399), and a Pilot Research Project Award from the University Cancer Research Fund at UNC Chapel Hill to JFR.

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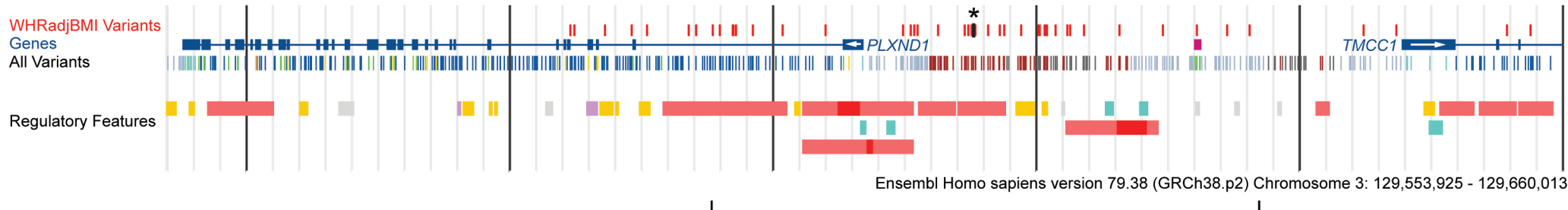
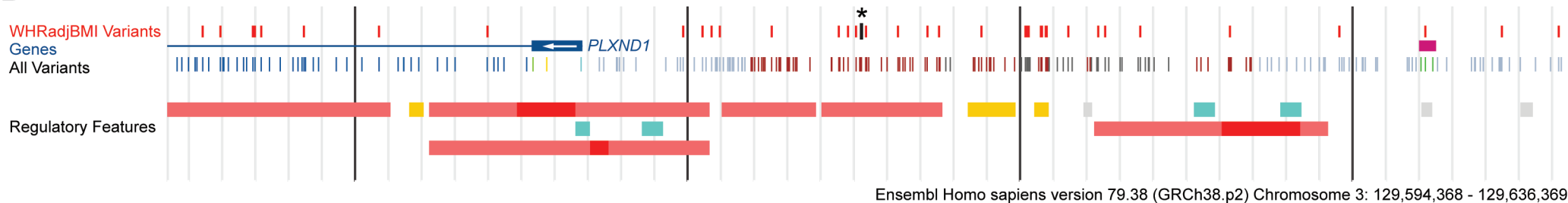
Figure Legends

Figure 1. Common variants and regulatory features at human *PLXND1*. Four tracks are depicted at the *PLXND1* locus. In descending order: WHRadjBMI Variants; 49 variants linked to rs10804591 (European ancestry, 1000G Phase 1, $>0.7 R^2$ in linkage disequilibrium of rs10804591). Genes; Havana annotated genes. Arrows within the first exon indicate direction of transcription. All Variants; common variants from 1000G Phase 1 with a frequency of at least 1% within populations of European ancestry. Regulatory Features; regulation marks predicted by the Ensembl Regulatory Build [66]. Vertical black bars are 100 kb (A) or 10kb (B) apart. Brackets in A denote the region shown in B.

Figure 2. β -coefficients and standard deviation for rs10804591. Bar charts indicate the β -coefficients and standard deviation (SD) for rs10804591 for waist-hip ratio adjusted for BMI (A; WHRadjBMI), waist circumference adjusted for BMI (WCadjBMI), and hip circumference adjusted for BMI (C, HIPadjBMI). All data are taken from Shungin et al. (2015). Asterisks indicate genome-wide significance ($P < 5 \times 10^{-8}$). Data are classified into 3 groups; sex-combined (black bars), female-only (white bars), and male-only (grey bars). Data are from GWAS or metabochip (MC) cohorts as described in Shungin et al. (2015).

Figure 3. Fluorescent lipophilic dyes to study body fat distribution in zebrafish. Nile Red stained zebrafish demonstrating neutral lipid stored within ATs (labelled in yellow) at two developmental stages. The arrows indicate VAT. SL = standard length (a measure of the fish length from the snout to the caudal peduncle).

Figure 4. Schematic illustrating the hypothesized mechanism by which vascular endothelial cell-derived *Plxnd1* determines ECM composition and VAT expandability. A. Overview of the hyperproliferative and hyperplastic microenvironment of *plxnd1* mutant zebrafish VAT. **B.** Schematic on the interaction between *Plxnd1*, Integrins and ECM composition.

A**B****All Variants Legend**

- Missense variant
- Intron variant
- Upstream gene variant
- Regulatory region variant
- Intergenic variant
- Non coding transcript exon variant
- ★ rs10804591

Regulatory Features Legend

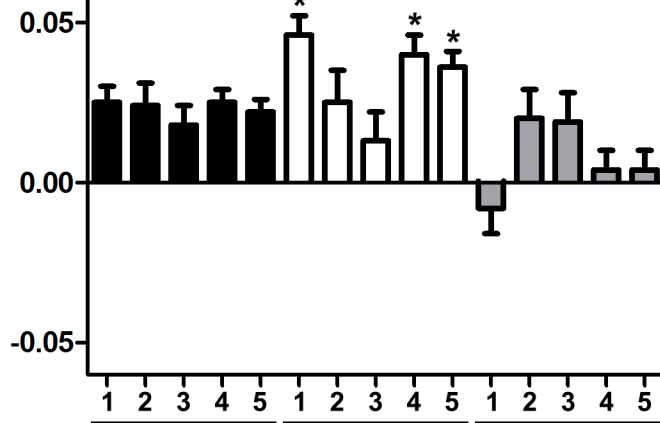
- Promoter
- Transcription Factor Binding Site
- Enhancer
- Open Chromatin
- Promoter Flank
- CTCF

A**WHRadjBMI**

sex-combined

female-only

male-only

 β -coefficient + SD

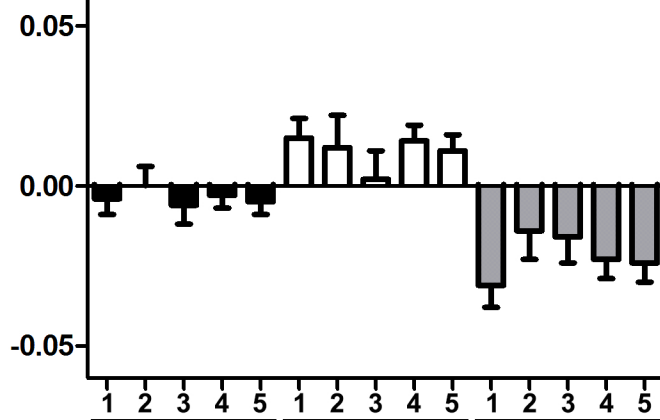
- 1 - GWAS (Euro-only)
- 2 - MC (Euro-only)
- 3 - MC (all-ancestries)
- 4 - GWAS + MC (Euro-only)
- 5 - GWAS + MC (all-ancestries)

B**WCadjBMI**

sex-combined

female-only

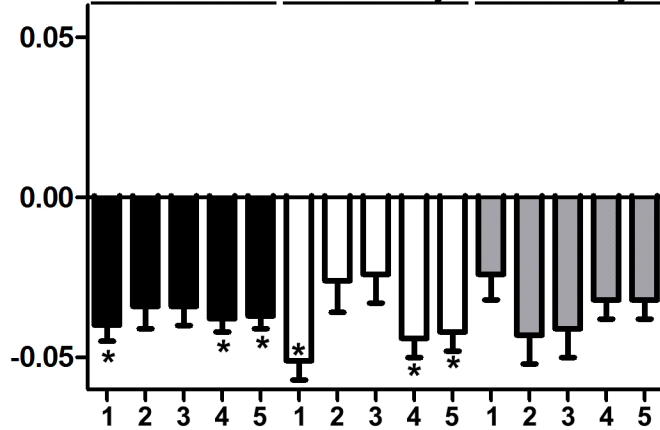
male-only

 β -coefficient + SD**C****HIPadjBMI**

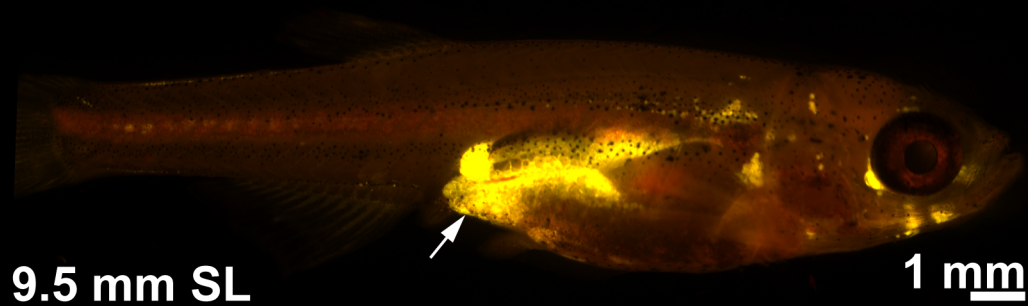
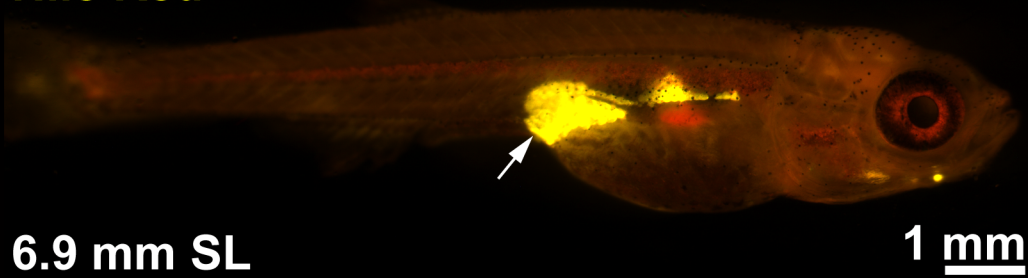
sex-combined

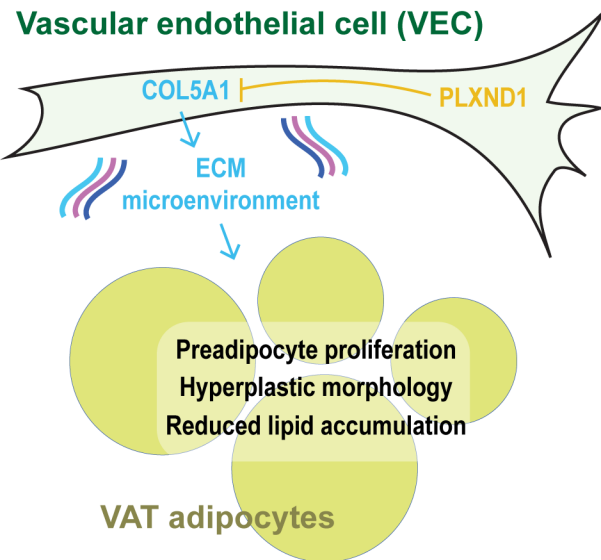
female-only

male-only

 β -coefficient + SD

Nile Red



A**Vascular endothelial cell (VEC)****B**